A Culture Medium and A Method for Detection of Parasites

Field of the Invention

This invention relates to a culture medium, a kit containing the culture medium and to a method for detection of a parasite such as *Dientamoeba fragilis* and/or another parasite.

Background

Dientamoeba fragilis (*D. fragilis*) is one of the most common parasites affecting animals including humans. It is thought to be spread via pinworms acquired by the faecal/oral route and resides in the gastrointestinal tract of the host, clinically causing such symptoms as abdominal discomfort, loose motions, bloating, diarrhoea, at times nausea, pruritus ani, malaise and other non specific symptoms. It is perhaps one of the most common parasites residing in the gastrointestinal tract of individuals in the western world and yet few physicians are aware of its presence and its contribution to disease, chiefly due to the fact that it is not diagnosed frequently.

Dientamoeba fragilis is notoriously difficult to diagnose unless suitable fixatives and permanent staining methods are employed and adequately trained personnel are available (Windsor and Johnson 1999 - Windsor JJ, Johnson EH. *Dientamoeba fragilis* : the unflagellated human flagellate. A review. Br J Biomed Sci 1999; 56:293-306). Culture methods have been shown to be more sensitive than microscopy at times (Ockert 1990, Sawangjaroen 1993 - Ockert G. Symptomatology, pathology, epidemiology and diagnosis of *Dientamoeba fragilis*: In : Honiberg BM, ed Trichomonads parasitic in humans. New York; Springer 1990; 394-410), however these culture methods are not currently used in diagnostic laboratories because of their complexity.

If the methodology could be simplified, culture would be more easy to perform and would have the added advantage that the isolates can be lysed and typed, thus aiding future epidemiological studies on top of simple diagnostic studies. *D fragilis* does not have a resistant cyst stage and consequently cannot survive outside the human host for longer than 12 hours (Sawangjaroen 1993 - Sawangjaroen N, Luke R, Provic P. Diagnosis by faecal culture of *Dientamoeba fragilis* in Australian patients with diarrhoea. Trans Roy Soc Trop Med Hyg 1993;87:163-5). In order for the culture method to be successful, the culture medium needs to be simple and needs to be one that will support the growth of *D. fragilis* and other parasites, eg *Blastocystis hominis*. Furthermore, the medium needs to have the features of long shelf life and transportability. In addition, the detection of the

growing parasites needs to be carried out easily by technicians with minimal training. Previous methods have included specific stains, eg., trichome and iron-haematoxylin, and more recently Riordan (US Patent No. 5,334,509) has suggested an acridine orange or acridine yellow stain for more specifically detecting *D. fragilis*. However, this method lacks specificity as it merely stains up RNA/DNA and therefore stains numerous parasites, including non-pathogenic ones. Using this method *D. fragilis* is at times indistinguishable microscopically from such parasites, and so the diagnosis again depends on the availability of highly trained microscopists to diagnose *D. fragilis*.

It would also be desirable to simplify the culture medium so that it can be used by an unskilled technician.

Summary of the Invention

It is the object of the present invention to overcome or substantially ameliorate at least one of the above described disadvantages.

According to a first aspect of the invention, there is provided a bi-phasic culture medium including

a solid phase containing an egg slope or agar slope; and

a liquid phase including a serum and a peptone.

In one embodiment, the solid phase is an egg slope.

In one embodiment, the serum is horse serum.

In one embodiment, the serum is rabbit serum.

In one embodiment, the peptone is bactopeptone.

In one embodiment, the liquid phase includes a phosphate buffered saline having a pH of from about 6.8 to about 7.8. Suitably the liquid phase contains up to about 98vol% of the phosphate buffered saline, about 1 to about 15vol% of the serum, and about 1 to about 15vol% of bactopeptone (about 1 to about 40w/w), in the liquid phase. The medium may also include an antibiotic from the class of macrolides, penicillins, cephalosporins, quinolones, aminoglycosides or other antibiotics. Suitably more than one antibiotic can be present. Suitable antibiotics include erythromycin, penicillin, streptomycin, clindamycin, cephalexin, vancomycin, rifampicin. Suitably a tetracycline is not used.

According to a second aspect, there is provided a kit including a container containing the medium according to the first aspect together with a container containing rice starch.

In one embodiment the kit includes a compartmentalized specimen bag. The kit may further include a utensil such as spoon or scoop for transferring a specimen such as

faecal matter into the container containing the medium. An additional container may also be provided for containing a specimen.

Suitably the container containing rice starch is a sachet.

According to a third aspect, there is provided a method of detecting the presence of a protozoa in a specimen, said method including

adding to the medium according to the first aspect, said specimen, rice starch and where necessary, an antibiotic,

allowing the medium to incubate for a time period so as to cultivate protozoa,

examining at least a portion of the incubated medium to detect the presence of protozoa.

Suitably the portion examined is or includes sediment.

According to a fourth aspect, there is provided a method of detecting protozoa in faecal matter, said method including adding to the medium according to the first aspect, faecal matter, rice starch and where necessary an antibiotic,

allowing the medium to incubate for a time period so as to cultivate intestinal protozoa,

examining at least a portion of the incubated medium to detect the presence of said protozoa.

Suitably the portion examined is or includes sediment.

Suitably the protozoa detected are one or more of *Dientamoeba fragilis*, *Blastocystis hominis*, *E. histolytica/dispar*, *Iodamoeba butschlii*, *Endolimax nana*, *Entamoeba coli*, or *Entamoeba hartmanni*. Most suitably *Dientamoeba fragilis*. Other suitably protozoa include protozoa of the genus referred to above, such as *Dientamoeba spp*, *Blastocystis spp*, *Entamoeba spp* or *Iodamoeba spp*. Suitably the portion of the sediment is examined microscopically, although a portion of the sediment can be stained and examined for various protozoa.

In one embodiment the medium is incubated for a period of up to 4 days. In another embodiment the medium is incubated for up to 48 hours. If desired, additional antibiotic and/or rice starch can be added during the incubation period such as after 24 hours of incubation. Suitably the medium is incubated at a temperature of about 36°C to about 38°C. For example, the temperature may be about 36°C, about 36.5°C, about 37°C, about 37.5°C, or about 38°C. Suitably the antibiotic is one or more antibiotics selected from the antibiotics listed above and is suitably one selected from the group consisting of erythromycin, penicillin, streptomycin, clindamycin, cephalexin, vancomycin and rifampicin.

Brief Description of the Drawing

Figure 1 shows a kit in accordance with one embodiment of the invention.

Description of Preferred Embodiments of the Invention

In the present invention the culture method and medium has been simplified to a less complex medium but one that will in use, support the growth of the protozoa referred above including *D fragilis*, *Blastocystis hominis* (*B. hominis*) and other parasites including other amoebae (for example *E. histolytica/dispar, Iodamoeba butschlii, Endolimax nana, Entamoeba coli, Entamoeba hartmanni* being other pathogens). The medium in accordance with the invention can double as a transport medium where a sample is taken off-site from the laboratory and then transported to the laboratory. The culture medium preferably uses an egg slope as opposed to previous used saline agar cultures. The egg slope may be made by any appropriate method known in the art, for example by diluting hen's eggs 50/50 in either Ringer's salt solution or PBS (phosphate buffered saline). The culture medium of the invention demonstrates enhanced reliability of culture. The medium is not as complex as those described in the prior art which comprise numerous chemicals. The medium in accordance with the present invention is – by culture standards – greatly simplified yet more reliable. Unlike the use of live *E. coli* bacteria (which are not suitable to be given to patients in order to collect their own specimens), the medium in accordance with the invention is designed to work even more reliably without *E.coli*.

In addition to the egg or agar slope, the culture medium also contains a liquid phase which includes a serum such as rabbit or horse serum and a peptone such as bactopeptone or bacteriological grade peptone. Typically the liquid phase which is suitably saline and suitably has a pH of from about 6.8 to about 7.8 (more typically about 7.4), contains the peptone in an amount of from about 1 to about 40vol%, preferably about 20vol%. Suitably in a 100ml formulation of the liquid phase, about 1 to 15mls, suitably about 5mls of the peptone, suitably a bactopeptone solution is used.

The present invention also provides a kit designed to allow a sample such as faecal matter to be placed immediately into the culture medium by the patient. One preferred form of a kit in accordance with the invention is shown in Figure 1. In Figure 1, the kit 1 may be in the form of a compartmentalized specimen bag 2 suitably containing four compartments. One compartment contains a specimen container 3 or bottle for collecting a sample such as faeces. A second compartment contains a utensil such as a scoop or spoon 4, suitably a plastic spoon. A third compartment contains a container 5, suitably a

sachet containing rice starch. A final container 6 containing the culture medium in accordance with the invention is provided in a fourth compartment.

In use, a patient collects a specimen such as faeces into the container 3. The faecal matter includes for example stool sample, lumina contents or colonoscopy aquired material. A small portion (suitably pea-size) of the specimen is then transferred by means of the scoop 4 and inoculated into the culture medium in container 6. The scoop can either then be discarded or resealed in the second compartment and discarded at the laboratory. Rice starch from container 5 is then added to the medium in container 6. Then, the culture medium is suitably transported to the laboratory for incubation while the protozoa such as *D fragilis* and other parasites survive the transportation due to the unique nature of the medium which can also double as a transport medium. A "pea-size" amount of stool is required only and it is placed into the culture medium with the contents of the enclosed sachet, added to make the transport/culture simplified. Left over faecal matter can be used for culture and sensitivity (C & S) and other parasites (parasite OVA).

The culture medium is bi-phasic and consists of a solid phase (the egg and/or agar slope) in a liquid phase. The liquid formulation per 100mls typically includes about 90mls Phosphate Buffered Saline pH 7.4 (suitable range about 6.8-7.8), about 5mls of serum, such as sterile Horse serum (suitable range about 1 – 15mls) and about 5mls 20% of peptone such as bactopeptone (suitable range about 1-15mls). Suitably about five drops (suitable range about 1 to 10 drops) of an antibiotic such as about 0.5wt% erythromycin (suitable range about 0.015wt% to 30wt%) is added to the culture medium and a small amount of rice starch for example an amount of about 10 mg to about 100 mg, is also added suitably from the sachet. Rice starch is essential for the xenic cultivation of intestinal protozoa (Clark and Diamond 2002 - Clark CG, Diamond LS. Methods for the cultivation of luminal parasitic protests of clinical importance. Clinical Microbiology Reviews 2002; 15:329-341). Once the specimen is added to the medium it is incubated at 37°C for 24 hours suitably in a laboratory (suitably at a temperature in the range of about 36°C to about 38°C). Then an extra two drops (suitable range 1 to 5 extra drops) of the antibiotic such as erythromycin are suitably added together with a small amount of rice starch, and a further incubation is suitably carried out for about 24 hours (suitably about 48 hours incubation in total) before examination suitably under a microscope is carried out. Further examinations may be carried out at 3 and finally at 4 days to allow for the occasional detection of slow-growing parasites which may include, for example one or more of *D. fragilis*, *B. hominis*, *E. histolytica/dispar*, *Iodamoeba butschlii*, *Endolimax nana*, *Entamoeba coli*, and *Entamoeba hartmanni*. When examined microscopically, a

drop of sediment is examined using the X20 objective of the microscope for the typical morphology of various protozoa. *D fragilis* ingests the rice starch voraciously, differentiating it from *B hominis* when viewed under the microscope. Under microscopic observation *D. fragilis* appear as round, refractile bodies packed with rice starch. Other intestinal amoebas such as *Entamoeba* and *Iodamoeba* also ingest rice starch, but *D fragilis* produces characteristic pseudopodia after 10-20 minutes at room temperature. These pseudopodia are leaf-like and are easily distinguishable from those produced by *Entamoeba*. Positive cultures can be simply confirmed by making a smear of the deposit, allowing it to air dry and fixing it in industrial methylated spirit or ethanol. This then can be stained with Giemsa (10% in PBS (phosphate buffered saline) pH6.8) for 20 minutes with a wash of buffer before examining under the microscope.

Other parasites and mixed infections may also be detected using the culture method of the invention. Any parasite growing in the culture can be identified by using simple stain. *Entamoeba* sp grow much larger than *D. fragilis* and the pseudopodia are much more obvious and larger. Any query regarding *E. histolytica/dispar* isolates can be resolved by lysing using 0.25ml 0.25% SDS containing 0.1 M EDTA and conducting a specific PCR/ELISA to confirm/exclude the pathogenic *E. histolytica*. Similarly, *B. hominis* can be detected using this culture. Although the pathogenicity of this parasite is controversial it has been associated with Irritable Bowel Syndrome. (IBS). It is possible that a certain subtype of *B. hominis* may be linked with disease, again a lysate can be made and then typed using riboprinting - Clark CG. Riboprinting: a tool for the study of genetic diversity in microorganisms. *J Euk Microbiol 1997; 44: 277-83.*

By use of the invention it is possible to detect protozoa including *D.fragilis* in a simple manner.

The invention will now be described with reference to the following examples

Clinical Examples

Example 1

In a 34y old female suffering with longstanding loose motions, wind and mild bloating a clinical diagnosis of "Irritable Bowel Syndrome" was made. To exclude enteric parasitic infestation, a stool test was ordered by the patient's physician. A faecal sample was collected by the patient using a small scoop provided in the kit in accordance with one embodiment of the present invention. A pea-sized amount of stool was placed into the culture medium of the invention, which in this case already contained the erythromycin. The contents of a sachet containing rice starch was also added. The

specimen was taken to the laboratory where it was incubated for 24 hours at 37°C. A small amount of rice starch was later added as well as 2 drops of erythromycin and the culture incubated a further 24 hours. A drop of the sediment was transferred onto a glass slide and a coverslip added. This preparation was examined under a light microscope using x20 objective. Round, refractile bodies that had ingested rice starch granules and produced delicate leaf-like pseudopodia after 10-20 minutes at room temperature were identified and a presumptive diagnosis of *D. fragilis* was made. A smear was made, allowed to air-dry, fixed in IMS (industrial methylated spirits) and was simply stained with Giesma for confirmation. The patient was treated with the appropriate anti-parasitic therapy and recovered.

Example 2

In a 49y old male patient with a family history of bowel malignancy, complaining of marked flatulence and hepatic flexure cramping pain, a colonoscopy was carried out to exclude the presence of bowel cancer. Simultaneously a sample of lumina contents was collected by aspiration. The colonoscopy acquired material was collected into the specialised faecal container of the invention and a small portion was transferred into the culture medium of the invention. The contents of a sachet provided in the kit in accordance with one embodiment of the invention containing rice starch was also added to the medium. The specimen was transported to the laboratory where it was incubated for 24 hours at 37°C. A further small amount of rice starch was then added together with 2 drops of erythromycin and the culture was incubated for a further 24 hours. A drop of the sediment was transferred onto a glass slide and a cover slip added. This preparation was examined under a light microscope using x20 objective. Round, refractile bodies that ingest rice starch granules and produce delicate leaf-like pseudopodia after 10-20 minutes at room temperature were detected and a presumptive diagnosis of *D. fragilis* was made. A smear was made, allowed to air-dry, fixed in industrial methylated spirits (IMS) and simply stained with Giemsa for confirmation.

Example 3

A female patient 39y of age presented with long standing gastrointestinal symptoms including abdominal pain, flatulence, distention, nausea and minimal weight loss. Repeated faecal samples were negative at the local hospital microbiology laboratory. In the most recent stool sample refractile bodies were observed using direct microscopy, although these could not be identified and did not show up in the parasite concentration method. The laboratory concerned did not use permanent faecal stains e.g. trichrome or

iron-haematoxylin. This is a common feature in Australia and the UK, where very few routine laboratories employ such methodologies. Part of the specimen was transferred into the culture medium of the present invention, using the small scoop provided within the kit of one embodiment of the invention. The contents of a sachet containing rice starch was also added to the medium. The specimen was transported to the laboratory where it was incubated for 24 hours at 37°C. A further small amount of rice starch was then added together with 2 drops of erythromycin and the culture was incubated for a further 24 hours. A drop of the sediment was transferred onto a glass slide and a cover slip added. The preparation was then examined under a light microscope using x20 objective. Microscopic analysis showed numerous refractile bodies, some of which ingested the rice starch. The Giemsa stain demonstrated the presence of both *D. fragilis* and *B. hominis*, a common finding in patients presenting with IBS-like symptoms.

In the above examples, detection is by standard methods using microscopy with special stains. The components of the formulations used can be sourced from many sources including Sigma Chemicals.

Although the invention is described in terms of various embodiments, it will be readily appreciated by those skilled in the art that various modifications, rearrangements and substitutions can be made without departing from the spirit of the invention.